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Anoxia tolerance and freeze tolerance in hatchling turtles

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Abstract Freezing survival in hatchling turtles may be limited by ischemic anoxia in frozen tissues and the associated accumulation of lactate and reactive oxygen species (ROS). To determine whether mechanisms for coping with anoxia are also important in freeze tolerance, we examined the association between capacities for freezing survival and anoxia tolerance in hatchlings of seven species of turtles. Tolerance to freezing (−2.5°C) was high in *Emydoidea blandingii*, *Chrysemys picta*, *Terrapene ornata*, and *Malaclemys terrapin* and low in *Graptemys geographica*, *Chelydra serpentina*, and *Trachemys scripta*. Hatchlings survived in a N_2 atmosphere at 4°C for periods ranging from 17 d (*M. terrapin*) to 50 d (*G. geographica*), but survival time was not associated with freeze tolerance. Lactate accumulated during both stresses, but plasma levels in frozen/thawed turtles were well below those found in anoxia-exposed animals. Activity of the antioxidant enzyme catalase in liver increased markedly with anoxia exposure in most species, but increased with freezing/thawing only in species with low freeze tolerance. Our results suggest that whereas oxygen deprivation occurs during somatic freezing, freeze tolerance is not limited by anoxia tolerance in hatchling turtles.

Keywords Freeze tolerance · Anoxia tolerance · Antioxidant · Lactate

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Introduction

The neonates of various species of freshwater turtles overwinter in terrestrial habitats where they may be exposed to subzero temperatures. Hatchlings can survive such exposure by supercooling (i.e., remaining unfrozen at temperatures below the tissue equilibrium freezing point) or by tolerating the freezing of their tissues (Costanzo et al. 1995). Natural freeze tolerance, which is also known in other reptiles and various terrestrial amphibians, requires an ability to survive the formation of ice within extracellular fluid compartments of the body (Storey et al. 1988).

During somatic freezing, hatchling turtles must cope with a host of physiological stresses associated with cellular dehydration, including osmotic/ionic perturbation and cell shrinkage. In addition, cardiac function and circulation cease during freezing (Rubinsky et al. 1994), resulting in a functional anoxia and associated lactacidosis (Churchill and Storey 1991, 1992a, 1992b; Packard and Packard 2004). Hatchling turtles vary in their tolerances to anoxia (Reese et al. 2004; Dinkelacker et al. 2005) and freezing (Costanzo et al. 1995; Packard et al. 1999), but whether or not these traits are associated in a manner suggesting that anoxia tolerance is a preadaptation to freeze tolerance has not been determined.

The link between anoxia tolerance and freeze tolerance has received only cursory attention, although Storey and Storey (1988) conjectured that the same mechanisms used by adult turtles to survive anoxia are used by hatchlings to survive freezing. During anoxia, adults cope with lactate accumulation by mobilizing calcium and magnesium carbonates from the shell and sequestering lactate in the shell (Jackson 2000). Nevertheless, death ultimately may result from an excessive fall in intracellular pH. Packard and Packard (2004) argued that freezing mortality in hatchling turtles results from lactic acidosis resulting from cardiovascular failure and the associated disruption of the buffering system.

Ischemic anoxia associated with somatic freezing could also result in an oxidative stress manifested as tissues reoxygenate after thawing (Hermes-Lima and Storey 1993). Oxidative stress is caused by the formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (OH·) (Hermes-Lima and Zenteno-Savin 2002). Superoxide and hydrogen peroxide are relatively unreactive; however, they can be quickly converted into the hydroxyl radical, which extensively damages macromolecules (Hermes-Lima et al. 1998). Some organisms increase antioxidant activities during or after anoxia exposure as a means to combat ROS produced upon reoxygenation (Hermes-Lima and Zenteno-Savin 2002), but whether hatchling turtles employ this defense mechanism in freezing or anoxia exposure is unknown.

Liver glycogen stores may contribute to the survival of turtles during somatic freezing and anoxia exposure. The products of glycogenolysis, glucose and lactate, may function as cryoprotectants to limit cell dehydration and reduce freezing damage to cells and intracellular structures, and glucose is a fermentable substrate supporting anaerobic production of ATP (Churchill and Storey 1991; Hemmings and Storey 2000). Intuitively, species maintaining large hepatic glycogen reserves, which may account for over one-half of the organ's mass (Hemmings and Storey 2000), may be predisposed to tolerating somatic freezing and anoxia exposure, although this supposition has not been tested in hatchling turtles.

The purpose of this study was to examine the association between capacities for anoxia tolerance and freeze tolerance among hatchlings of turtle species that vary in these traits. Toward this end, we compared measures of survival and physiological responses to somatic freezing and anoxia exposure. Taken together, our results help clarify the fundamental role of anoxia tolerance in the evolutionary development of vertebrate freeze tolerance.

Materials and methods

Turtles were hatched from eggs obtained from gravid females in Nebraska [snapping turtles (*Chelydra serpentina*), ornate box turtles (*Terrapene ornata*), painted turtles (*Chrysemys picta bellii*), and Blanding's turtles (*Emydoidea blandingii*)], Indiana [red-eared sliders (*Trachemys scripta*) and northern map turtles (*Graptemys geographica*)], and New Jersey [diamondback terrapins (*Malaclemys terrapin*)]. Eggs were incubated at 28°C on moist vermiculite (1.0 g water g^{-1} dry vermiculite) until they hatched in August and then were cold acclimated following the procedure described by Costanzo et al. (2000). Briefly, hatchlings were held on moist vermiculite in plastic boxes kept in a darkened incubator (Model I-35X, Percival, Boone, IA, USA). The incubator temperature was held at 20°C during September, but was lowered to 15°C on 1 October and then to 10°C on 1 November. Hatchlings were kept at 4°C from 1 December until they were used in the experiments, in February. They were denied food and water throughout the experiment because hatchlings do not feed during the winter.

Freeze tolerance trials

We prepared hatchlings for testing by weighing them, wetting them with cold water, and placing them vertically in a plastic centrifuge tube immersed in a refrigerated ethanol bath (Model RTE 140, Neslab, Portsmouth, N. H.). A copper-constantan thermocouple placed against the body of turtle allowed us to record temperature on a chart recorder throughout the experiment. Turtles were permitted to supercool slightly (to −1.2°C) before we initiated somatic freezing by touching the wet skin of the neck with a frozen cotton swab. Upon appearance of a freezing exotherm, we inserted an insulating foam plug into the tube directly above the turtle. This inoculation procedure eliminated the risk of a rapid, uncontrolled freezing event that is potentially fatal (Storey and Storey 1988). We allowed the heat of fusion to dissipate and the hatchlings to attain thermoequilibrium at −1.2°C before further cooling them at 0.05°C h^{-1} to the target temperature, −2.5°C. Turtles (n = 4–8 per species) were held at −2.5°C for 3 d or 7 d and then were removed from the bath, placed on a moist paper towel inside individual plastic cups, and thawed at 4°C. Most species were tested using both freeze durations in order to better characterize their capacity for freeze tolerance.

Costanzo et al. (2004) reported that freezing-exposed turtles can require up to 6 d after thawing to exhibit normal neuromuscular reflexes. To be cautious, we determined the survival status of each turtle by examining its response to tactile stimulation (gently pressing the limbs with a blunt probe) 14 d after thawing. Turtles ultimately scored "alive" were quickly responsive to the stimulus, alert, and often mobile, whereas those that succumbed to freezing were unresponsive and had flaccid appendages that extended from the body. We categorized the freeze tolerance capacity of each species as "low" or "high" on the basis of whether at least one-half of the turtles in the sample recovered from a 7-d freezing trial.

Anoxia tolerance trials

Hatchlings were tested for anoxia tolerance in a covered plastic chamber (volume, ~2080 cm^3) that received a continuous flow of N_2 gas. Small access holes were installed in the chamber lids and sealed with rubber plugs. The trials were conducted in darkness at 4°C, and moist sponges were placed inside the chamber to maintain high humidity and inhibit dehydration of the hatchlings. Samples of chamber gas that were tested with an oxygen analyzer (Model S-3A/II, Ametek, Pittsburgh, Penn.) on

several occasions during the trial showed that the chamber essentially was anoxic (average, 0.9% O_2; range, 0.7–1.2%). We examined the hatchlings (n = 6–8 per group) daily to assess their survival status, using the same viability criterion as used in the freeze-tolerance trials (i.e., response to tactile stimulation). Care was taken to minimize gas exchange and exposure to light during the examinations. Turtles remained inside the chamber until all had died; time of death of each individual was recorded to the nearest day.

Physiological responses to freezing and anoxia

We compared plasma lactate concentration, liver catalase activity, and characteristics of the liver (wet mass, dry mass, and water content) among control, frozen/thawed, and anoxia-exposed hatchlings. Control turtles (n = 5–8 per species) were sampled directly after removing them from their cages at 4°C, whereas other hatchlings were frozen to −2.5°C for 2 d (n = 7–8 per species) or exposed to anoxic conditions for 16 d (n = 5–8 per species), as described previously. Before sampling the blood and liver, we permitted frozen hatchlings to thaw and recover at 4°C for 24 h and held anoxia-exposed turtles in air at 4°C for 30 min, assuming that ROS formation and the risk of oxidative stress would accompany reoxygenation of the tissues. We did not collect physiological data for frozen/thawed *G. geographica* and *T. ornata* because too few animals were available for study.

Hatchlings were euthanized by severing the spinal cord and blood was collected directly from the severed carotid arteries into heparinized microhematocrit tubes, which were centrifuged at 2,000 *g* for 5 min. The plasma was stored at −20°C in plastic vials until analyzed for lactate concentration (Sigma, no. 735, St. Louis, Mo.). The liver was dissected from the carcass, weighed, and divided into two portions. To measure catalase activity, a portion (~80 mg) of the liver was mechanically homogenized in 1.0 ml of extraction buffer (10 mM HEPES, 70 mM sucrose, 200 mM mannitol, 1 mM EGTA; pH 7.5) for 30 s and then centrifuged at 700 *g* for 5 min. The supernatant was removed and analyzed for catalase activity (Sigma, no. CAT100), defined as the amount of enzyme decomposing 1 μmol H_2O_2 min^{-1} at 20°C. Soluble protein was measured using the Bio-Rad prepared reagent and bovine serum albumin as the standard. The remaining portion of liver was weighed and dried in a 65°C-oven for 72 h. We determined the amount of water in the sample by subtracting the dry tissue mass from the wet tissue mass. Dry mass of the whole organ was estimated by multiplying the percent dry mass by the fresh mass of the intact liver.

Statistical analyses

Mean survival times of anoxia-exposed turtles were compared among species using analysis of variance (ANOVA, Bonferroni multiple comparisons). ANOVAs were used to compare within-species variation in plasma lactate concentration and liver catalase activity among control, frozen/thawed, and anoxia-exposed turtles. Liver characteristics were compared among species using analysis of covariance (ANCOVA), with body mass as the covariate; percentage data were arcsine-square root transformed prior to analysis. Linear regression analyses were used to examine the relationships among lactate accumulation rate, lactate concentration, and anoxia survival times. Significance was judged at $P \leq 0.05$.

We eschewed phylogenetically-based methods for comparing capacities for freeze tolerance and anoxia tolerance among turtle species for two reasons. First, the ambiguities in our present knowledge of emydid phylogeny (Stephens and Wiens 2003) confound determination of accurate branch lengths, which are necessary for constructing phylogenetically-independent contrasts (Garland et al. 1992). Second, previous studies of adult turtles attest that anoxia tolerance has strong ecological, but not phylogenetic, associations (Ultsch and Jackson 1995; Reese et al. 2003).

Results

Tolerance of somatic freezing varied markedly among the species we examined (Table 1). At least some individuals of every species tolerated 3 d of freezing at −2.5°C. However, because only one half of the *G. geographica* survived a 3-d exposure, and because *T. scripta*

Table 1 Survival of hatchling turtles following freezing at −2.5°C for 3 or 7 d, or anoxia exposure at 4°C

Species	Freezing survival (no. survived/no. tested)		Freeze tolerance capacity	Anoxia Survival time (d)	n
	3 d	7 d			
Graptemys geographica	2/4	n.d.	low	50 ± 2[a]	(6)
Chelydra serpentina	5/8	1/8	low	27 ± 3[b]	(8)
Trachemys scripta	5/8	0/8	low	27 ± 2[b]	(8)
Chrysemys picta	n.d.	7/8	high	41 ± 3[ac]	(8)
Emydoidea blandingii	n.d.	7/8	high	35 ± 4[bc]	(8)
Malaclemys terrapin	8/8	8/8	high	17 ± 1[d]	(8)
Terrapene ornata	6/6	5/6	high	39 ± 7[c]	(6)

Survival times are means ± SEM. Mean values not sharing a letter were statistically distinguishable ($P < 0.05$). n.d., not determined

and *C. serpentina* fared poorly in the 7-d freezing trials, these species were characterized as having a low freeze tolerance capacity. In contrast, nearly all individuals of *T. ornata*, *C. picta*, *E. blandingii*, and *M. terrapin* recovered after 7 d of freezing and thus freeze tolerance in species were characterized as being high.

Anoxia tolerance also varied among species (Table 1). Mean survival times for anoxia-exposed turtles ranged from 17 d for *M. terrapin* to 50 d for *G. geographica*. Survival times for *T. scripta* and *C. serpentina* were similar, and lower than those determined for *T. ornata*, *C. picta*, and *E. blandingii*.

Plasma lactate concentrations were uniformly low (0.4–3.5 mmol l^{-1}) in control animals and ~20-fold higher than controls in the frozen/thawed turtles (Fig. 1). We found no clear association between freeze tolerance capacity and the amount of lactate in the blood of frozen/thawed turtles. Notably, the highest and lowest lactate concentrations were found in the highly freeze-tolerant species *C. picta* (27.6 ± 1.7 mmol l^{-1}) and *M. terrapin* (15.7 ± 4.5 mmol l^{-1}), respectively.

Lactate concentrations in anoxia-exposed turtles were generally 71-fold (range = 22- to 200-fold) higher than those measured in control animals and about 4-fold (range = 3- to 6-fold) higher than those in frozen/thawed turtles (Fig. 1). Rates of lactate accumulation in anoxia-exposed turtles, expressed as mmol l^{-1} d^{-1}, varied among species; both the highest and lowest rates were found in the highly freeze tolerant species *M. terrapin* (6.2 ± 0.2 mmol l^{-1} d^{-1}) and *T. ornata* (4.0 ± 0.1 mmol l^{-1} d^{-1}), respectively. Lactate accumulation rate and survival time in anoxia varied inversely to each other, related, although the trend lacked statistical significance (Fig. 2).

Among the control animals, liver mass (after adjustment for covariation with body size) varied interspecifically, ranging from 1.4% (*C. serpentina*) to

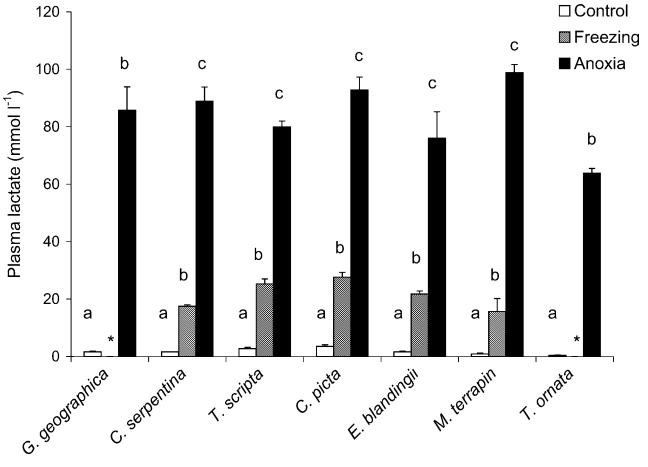

Fig. 1 Plasma lactate concentrations in control, frozen/thawed, and anoxia-exposed hatchling turtles. All values are means ± SEM (n = 5–8 per group). Within a species, values identified by different letters were statistically distinguishable ($P < 0.05$). Missing data are indicated by an asterisk

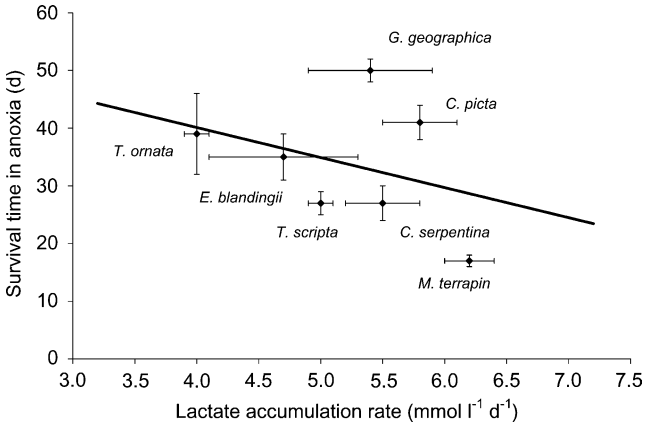

Fig. 2 Relationship between survival time during anoxia exposure and rate of lactate accumulation in hatchling turtles. All values are means ± SEM (n = 6–8 for survival time; n = 5–8 for lactate accumulation rate). Mean values were fitted by linear regression ($F_{1,5} = 0.70$, $P = 0.44$, $r^2 = 0.12$)

3.7% (*T. scripta*) of total body mass. Livers excised from frozen/thawed turtles typically were ~10% heavier than those in conspecific control animals, although the difference was statistically significant only for *C. serpentina* and *E. blandingii* (Table 2). The apparent increase in liver mass with freezing/thawing (see also Hemmings and Storey 2000) likely reflected tissue hyperhydration, as the livers of these hatchlings contained more water (range: 68.2% in *M. terrapin* to 78.4% in *E. blandingii*) than the livers of control animals (range: 63.0% in *M. terrapin* to 73.6% in *C. serpentina*). Dry liver mass was similar between the control and frozen/thawed turtles, but was about 19% less in the anoxia-exposed turtles. The livers of anoxia-exposed turtles also tended to contain more water (range: 66.7% in *M. terrapin* to 78.0% in *E. blandingii*) than the livers of control animals (Table 2).

Liver catalase activity in control animals varied ($F_{6,42} = 8.1$, $P = 0.0001$) among species, with mean values ranging from 106 to 369 U mg^{-1} protein (Fig. 3). Relative to the controls, enzyme activity was 2- to 3-fold higher in frozen/thawed *C. serpentina* and *T. scripta*. Mean values were nominally higher in frozen/thawed *C. picta* and *E. blandingii*, but the difference from control lacked statistical significance. Liver catalase activity in anoxia-exposed turtles exceeded that in the corresponding controls in five of the seven species examined (Fig. 3). With one exception (*M. terrapin*), enzyme activities in frozen/thawed and anoxia-exposed turtles were similar. However, whereas catalase activity increased under stress in some species (*C. serpentina*, *T. scripta*, *C. picta*, *M. terrapin*), in others (*G. geographica*, *E. blandingii*, *T. ornata*) the response was modest or lacking.

Discussion

The ability to recover from somatic freezing varied considerably among the seven species of turtles we

Table 2 Liver characteristics among species of hatchling turtles following freezing at −2.5°C for 2 d or anoxia exposure at 4°C for 16 d

Species	Liver Wet Mass (mg)	Liver Dry Mass (mg)	Water Content (% wet mass)
Graptemys geographica			
Control	191.3 ± 16.2 (5)^{A}	68.3 ± 6.2 (5)^{A}	64.4 ± 0.8 (5)^{A}
Anoxic	194.8 ± 14.8 (5)^{A}	61.7 ± 5.0 (5)^{A}	68.4 ± 0.4 (5)^{B}
Freezing	n.d.	n.d.	n.d.
Chelydra serpentina			
Control	129.9 ± 11.1 (8)^{A}	34.7 ± 3.6 (8)^{A}	73.6 ± 0.7 (8)^{A}
Anoxic	147.3 ± 9.3 (8)^{AB}	33.6 ± 2.0 (8)^{A}	77.2 ± 0.6 (8)^{B}
Freezing	170.2 ± 8.8 (7)^{B}	39.8 ± 2.2 (7)^{A}	76.6 ± 0.9 (7)^{B}
Trachemys scripta			
Control	263.9 ± 11.0 (8)^{A}	97.3 ± 4.5 (8)^{A}	63.2 ± 0.5 (8)^{A}
Anoxic	225.7 ± 13.6 (8)^{B}	73.0 ± 4.3 (8)^{B}	67.6 ± 0.4 (8)^{B}
Freezing	287.9 ± 11.3 (8)^{A}	88.0 ± 4.0 (8)^{A}	69.5 ± 0.4 (8)^{C}
Chrysemys picta			
Control	133.0 ± 14.1 (8)^{A}	43.1 ± 4.8 (8)^{A}	67.7 ± 0.9 (8)^{A}
Anoxic	130.4 ± 13.3 (8)^{A}	36.6 ± 4.4 (8)^{A}	72.4 ± 1.0 (8)^{B}
Freezing	152.5 ± 10.4 (8)^{A}	40.6 ± 2.9 (8)^{A}	73.4 ± 0.5 (8)^{B}
Emydoidea blandingii			
Control	151.6 ± 10.3 (8)^{A}	47.8 ± 8.1 (8)^{A}	69.3 ± 3.6 (8)^{A}
Anoxic	161.5 ± 5.0 (8)^{A}	35.5 ± 1.4 (8)^{B}	77.9 ± 0.8 (8)^{B}
Freezing	197.6 ± 15.2 (8)^{B}	42.8 ± 3.7 (8)^{AB}	78.4 ± 0.4 (8)^{B}
Malaclemys terrapin			
Control	188.3 ± 12.3 (8)^{AB}	69.8 ± 5.3 (8)^{A}	63.0 ± 0.8 (8)^{A}
Anoxic	181.4 ± 13.9 (8)^{A}	60.9 ± 6.4 (8)^{A}	66.7 ± 1.1 (8)^{B}
Freezing	220.4 ± 12.1 (8)^{B}	69.7 ± 3.8 (8)^{A}	68.3 ± 0.8 (8)^{B}
Terrapene ornata			
Control	283.9 ± 25.7 (5)^{A}	99.8 ± 13.0 (5)^{A}	64.6 ± 4.4 (5)^{A}
Anoxic	233.7 ± 32.6 (5)^{A}	68.6 ± 11.0 (5)^{A}	71.0 ± 1.0 (5)^{A}
Freezing	n.d.	n.d.	n.d.

Values are means ± SEM. Mean values not sharing a letter were statistically distinguishable ($P < 0.05$). n.d., not determined

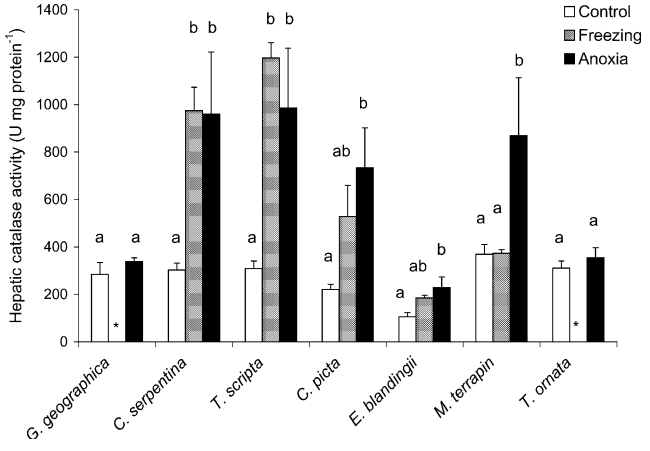

Fig. 3 Liver catalase activity in control, frozen/thawed, and anoxia-exposed hatchling turtles. All values are means ± SEM (n = 5–8 per group). Within a species, values identified by different letters were statistically distinguishable ($P < 0.05$). Missing data are indicated by an asterisk

examined. Our results confirm earlier reports of profound freeze tolerance in hatchling *C. picta* (Storey et al. 1988; Churchill and Storey 1991, 1992a), *E. blandingii* (Dinkelacker et al. 2004), and *T. ornata* (Costanzo et al. 1995) and also corroborate a recent finding (Baker et al. 2004) that hatchling *M. terrapin* tolerate an extended period of freezing at temperatures near −2.5°C. Furthermore, our results confirm that freeze tolerance is poorly developed in hatchling *C. serpentina* (Costanzo et al. 1995; Packard et al. 1999), and *T. scripta* (Churchill and Storey 1992b; Packard et al. 1999). Two of the four *G. geographica* survived in our freeze-tolerance trials; however, we categorized this species as having low freeze tolerance. Our conclusion concurs with Baker et al. (2003) who reported that hatchling *G. geographica* did not recover after being held frozen at −2.5°C for as few as 24 h. Our present data, together with the findings of these earlier studies, refute the hypothesis advanced by Packard et al. (1999) that the ability to withstand freezing is widespread among the hatchlings of North American turtles and is a shared ancestral trait in chelonians.

For the purposes of our analyses, we categorized the freeze tolerance capacity of various turtle species simply as "low" or "high" on the basis of their responses to our experimental freeze/thaw protocol. Nevertheless, the outcome suggests an interesting association between our assessment and the hibernation ecology of these animals. For example, species exhibiting the higher level of freeze tolerance are known (*C. picta*, *M. terrapin*, and *T. ornata*) or suspected (*E. blandingii*) to hibernate in terrestrial microenvironments where frost may occur (Costanzo et al. 1995, 2004; Dinkelacker et al. 2004). Conversely, species characterized as poorly freeze tolerant commonly overwinter where frost is uncommon (*T. scripta* and *C. serpentina*: Costanzo et al. 1995; Packard et al. 1999) or are adept at avoiding freezing through supercooling (*G. geographica*: Baker et al. 2003).

The length of time hatchling turtles survived in anoxia varied among species, indicating that some taxa

are better prepared physiologically to cope with the attendant stresses. Hatchling turtles tolerate anoxia less well than adults, probably owing to their limited buffer reserves and their inability to sequester lactate within the shell (Reese et al. 2004; Dinkelacker et al. 2005; Ultsch and Reese 2005).

Variation in anoxia tolerance among the hatchlings of different species also could reflect differences in buffer reserves and metabolic rates (Reese et al. 2004; Dinkelacker et al. 2005). The limited anoxia tolerance among hatchling turtles found in the present study generally concurs with the results of Reese et al. (2004), who reported that hatchlings representing three species of turtles survived 10–30 d in a N_2 atmosphere. Of the seven species we examined, *G. geographica* was the most tolerant, surviving an average of 50 d in anoxia. In contrast, Reese et al. (2004) reported that *G. geographica* survived only 10 d in anoxia, a finding that better accords with the perception that adult *G. geographica* are comparatively anoxia intolerant and restricted to aerobic habitats (Crocker et al. 2000; Reese et al. 2001). However, we have no cause to doubt the validity of our data for this species. Various methodological differences (e.g., feeding and acclimation regimes) between the studies might account for this discrepancy.

Physiological responses to freezing and anoxia

Previous studies have shown that somatic freezing elevates lactate concentrations in the blood and organs of hatchling *C. picta* (Storey et al. 1988; Churchill and Storey 1991, 1992a; Packard and Packard 2004) and *T. scripta* (Churchill and Storey 1992b). Our present results not only confirm this finding for these two species, but extend it to five other taxa. Furthermore, they suggest that lactate accumulation is a generalized response to freezing in hatchling turtles. Lactate accumulation during freezing probably reflects a greater reliance on anaerobic production of ATP once breathing ceases and circulation fails (Storey et al. 1988). Anaerobic glycolysis is also key to ATP production during periods of environmental anoxia and, not surprisingly, lactate accumulated during anoxia exposure in every species that we examined. Substantially higher levels of plasma lactate were attained in anoxia-exposed turtles (64–99 mmol l^{-1}) as compared to the animals subjected to freezing (Fig. 1). The variation in this response could reflect differences in the exposure temperature (−2.5°C versus 4°C) or exposure duration (2 d versus 16 d), or the possibility that turtles were active whilst inside the anoxia chambers.

Churchill and Storey (1991) suggested that lactate could serve a cryoprotectant role in *C. picta*, a highly freeze-tolerant species. Our data indicate that lactate is not accumulated specifically for such purpose, as the response was also seen in poorly freeze-tolerant species, such as *C. serpentina* and *T. scripta*. Furthermore, it is questionable whether the amount of lactate accumulated during freezing (commonly < 25 mmol l^{-1} ; 17 to 28 mmol l^{-1} in the present study) could have a significant colligative effect in reducing cellular dehydration. Packard and Packard (2004) conjectured that accumulated lactate (~2 mg g^{-1}) directly causes mortality in freezing-exposed hatchling *C. picta*. The findings of Reese et al. (2004) rather suggest that these turtles can tolerate a much higher lactemia, as they achieved a lactate concentration of 3.6 mg g^{-1} within the first 10 d of anoxia exposure yet survived another 20 d. Therefore, the limit of freezing survival probably is reached well before lactacidosis becomes critical and more likely stems from physical damage by ice crystals and osmotic insult to membranes and macromolecules (Storey et al. 1988).

Lactate accumulation and the associated fall in intracellular pH is an important constraint in anoxia tolerance in turtles and other organisms. In a study of adult turtles, Reese et al. (2002) found that relatively anoxia-intolerant species tended to accumulate lactate faster (~2-fold) than more tolerant species. Our present results for hatchling turtles exhibited a similar trend, although the relationship was not statistically significant owing at least in part to the surprisingly high anoxia tolerance of *G. geographica* (Fig. 2). Reese et al. (2004) found no variability in lactate accumulation rates among anoxia-exposed hatchling *C. picta*, *C. serpentina*, and *G. geographica* even though their survival times varied considerably. However, in this study lactate concentrations at the survival limit differed markedly among the taxa. Similarly, Dinkelacker et al. (2005) found that hatchling *C. serpentina* survived longer under hypoxic submergence than did *E. blandingii* and accumulated as much lactate in 19 d as *E. blandingii* did in 13 d. Regarding our present data, a post hoc analysis in which we estimated the plasma lactate concentrations at death by extrapolating from measured rates of accumulation, showed that survival time in anoxia is directly correlated ($F_{1,5} = 24.2$, $P = 0.004$, $r^2 = 0.83$) with lactemia. Therefore, interspecific variation in anoxia tolerance among hatchling turtles probably is associated with their ability to tolerate or mitigate the effects of a large lactate load, although differences in metabolic rate and buffering capacity also may be important (Dinkelacker et al. 2005; Reese et al. 2004).

Given the importance of glycogen in anaerobic glycolysis and the fact that stored glycogen can comprise nearly one-half of the dry mass of liver (Hemmings and Storey 2000), we anticipated that species with larger livers would better tolerate freezing and anoxia. To the contrary, *T. scripta* had the largest liver but was poorly freeze tolerant, and *E. blandingii*, which had the smallest liver, was among the most anoxia tolerant of the group (Table 2). An ample liver glycogen reserve is critical to anoxia survival because hatchling turtles submerged in anoxic water utilize this substrate at rates 10-fold higher than do hatchlings submerged in normoxic water; however, the size of glycogen supply probably is not limiting under most circumstances because 33–50% of

the initial amount remains at the limit of anoxia survival (Reese et al. 2004). Regardless, the substantial decrease in liver dry mass during anoxia exposure is probably due to glycogen depletion (Table 2).

Oxidative stress in freezing and anoxia

Oxidative stress occurs whenever ROS production in the mitochondrial respiratory chain overtaxes the various antioxidant defense mechanisms and, not surprisingly, this scenario commonly arises during recovery from a reduced metabolic state. In order to combat increased ROS generation, some animals rely on antioxidant defense enzymes, such as catalase, which decomposes hydrogen peroxide. Maintaining high constitutive levels of antioxidant enzyme activity is a defensive strategy employed by species prone to oxidative stress (Joanisse and Storey 1996a; Willmore and Storey 1997). The relatively high levels of hepatic catalase activity in our control animals (Fig. 3) suggest that hatchling turtles may belong to this group. An alternative strategy is to upregulate antioxidant defenses either in anticipation of, or in direct response to, increasing levels of ROS production (Hermes-Lima and Storey 1993; Hermes-Lima and Zenteno-Savin 2002; Ferreira et al. 2003) and this response also occurred in some of the species recovering from somatic freezing and/or anoxia exposure.

Whereas liver catalase activity increased in most species in response to anoxia, freezing/thawing caused significant elevations only in *C. serpentina* and *T. scripta* (Fig. 3). This difference could indicate that oxygen deprivation during the 2-d freezing exposure was too brief to cause significant ROS production (Rifkind et al. 1993), that constitutive levels of catalase activity were sufficient (Willmore and Storey 1997), or that frozen/thawed turtles instead rely on peroxidase enzymes, ascorbate, or GSH to detoxify H_2O_2 (Fleck et al. 2003). Somatic freezing and anoxia exposure stimulate different antioxidant defense systems in the garter snake, *Thamnophis sirtalis* (Hermes-Lima and Storey, 1993) and larvae of the goldenrod gall fly, *Eurosta solidaginis* (Joanisse and Storey 1998), and recent study of hatchling *T. scripta* has shown that these stresses differentially impact mitogen-activated protein kinases mediating cellular responses, despite the fact that ischemic anoxia is one of the component stresses of somatic freezing (Greenway and Storey 1999).

Hatchlings of species exhibiting well-developed freeze tolerance did not express increased liver catalase activity during recovery from freezing/thawing, whereas poorly freeze-tolerant species did. If one interprets an upregulation of antioxidant enzyme activity following oxygen deprivation as providing a greater capacity for the removal of ROS, then our results for hatchling turtles appear counter-intuitive. However, these specially-adapted species might tolerate higher levels of free radical damage or otherwise enhance mechanisms for removing damage products (Willmore and Storey 1997a). Our results are reminiscent of the case in which antioxidant enzymes increased during winter in freeze-intolerant, but not freeze-tolerant, insects (Joanisse and Storey 1996b). Additional information regarding ROS formation and other antioxidant activities is needed to elucidate the relationship between freeze tolerance capacity and antioxidant defenses.

Links between freeze tolerance and anoxia tolerance

Our results for hatchling turtles fail to support the hypothesis that capacities for tolerating somatic freezing and anoxia exposure are positively correlated (Fig. 4). The expected relationship was upset by the responses of *G. geographica*, which survived in an N_2 atmosphere much longer than expected, and *M. terrapin*, which exhibited surprisingly low anoxia tolerance. This outcome indicates that anoxia tolerance is not a factor limiting freezing survival, an interpretation consistent with our finding that highly freeze-tolerant species do not accumulate lactate or mobilize antioxidant defenses under freeze/thaw stress to the extent they do under anoxia stress. However, we emphasize that this conclusion does not imply that anoxia stress is irrelevant to freezing survival, but, contrary to the claim by Packard and Packard (2004), only suggests that it is not limiting.

With the exception of *C. serpentina*, all of the species we examined are grouped within the Emydidae, so our findings reveal little about the phylogenetic associations between anoxia tolerance and freeze tolerance capacity in turtles. Emydids show variability in capacities for both freeze tolerance (Baker et al. 2003) and anoxia tolerance (Reese et al. 2004; Dinkelacker et al. 2005). Much less attention has been devoted to turtles of other families, but the available evidence suggests that members of the Trionychidae, Kinosternidae, and Chelydridae exhibit low tolerance to somatic freezing (Costanzo et al. 1995; Packard et al. 1999) and/or anoxia (Reese

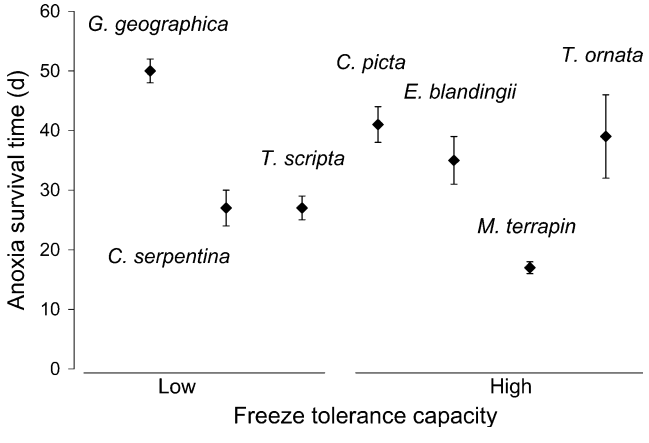

Fig. 4 Association between capacity for freeze tolerance and anoxia survival time among hatchlings of seven species of turtles. All values are means ± SEM (n = 6–8 per group)

et al. 2004; Dinkelacker et al. 2005). Additional study will be needed to more fully understand the mechanisms of evolution of environmental stress tolerances among chelonians.

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